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METHODS AND TEST KITS FOR EVALUATING THE PRESENCE AND SEVERITY  
OF RESPIRATORY TRACT INFLAMMATION

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The Technical Field of the Invention

The present invention is related to methods and test kits for evaluating the presence and severity of inflammation in respiratory tracts. The methods and kits can be used for evaluating the level of the inflammatory lung tissue injury, predicting and preventing complications and the risk of an acute inflammation from turning into a chronic process, targeting the treatment modalities to reduce tissue destruction and evaluating the efficacy of an applied therapy. The diagnosis is preferably performed as a rapid and reliable chair-side assay using human and/or animal respiratory secretion samples representing whole and/or part of lung. The methods and test kits are based on the use of one or more binding substances capable of specifically recognizing one or more matrix metalloproteinases (MMPs) and/or related molecules (MMP-RMs) alone or in combination from said respiratory secretion samples.

The Background of the Invention

Airways are daily exposed to various pollutants and airborne particles, which are suspected to be incriminates in the onset and persistence of respiratory airborne diseases. Individual expose by cigarette smoke increases dramatically an influx of damaging agents into airways of smokers. Lung disorders occurring with airflow limitation include bronchial asthma, chronic obstructive pulmonary disease (COPD), chronic bronchitis and bronchiectasis in man.

In addition to obstructive syndrome, said pulmonary diseases are connected with a common denominator which is various levels of chronic inflammation in the airways and lung tissue. Chronic disorders and every inflammatory exacerbation in the

airways leads to tissue injury and consequent worsening of the maintenance of either structural or functional integrity of the lung.

Chronic inflammatory lung diseases like chronic obstructive pulmonary disease (COPD), bronchial asthma, bronchiectasis and chronic bronchitis are characterized by re-occurring bursts of inflammation leading to increased mucus production, epithelial injury and constriction of bronchial wall. Long lasting inflammation or several inflammatory bursts finally lead to fibrosis and to permanent dysfunction of the whole respiratory system. Alternatively, lung inflammation and tissue destruction can result from viral, bacterial, microbial and/or fungal infections.

Conventionally, the diagnosis of chronic respiratory diseases in man is based on clinical examination, radiological findings, lung function testings, cytology of sputum or the epithelial lining fluid (ELF) as well as morphology of biopsies from respiratory tract.

Diagnostics of equine chronic obstructive pulmonary disease (COPD) is based on presence of chronic cough or laboured breathing in an otherwise healthy animals after exclusion of other conditions, mainly infectious diseases involving lower airways. In equine COPD the complete blood count remains normal in the absence of concurrent infection. In early stages of COPD respiratory secretion samples obtained by bronchoalveolar lavage (BAL) are needed in order to confirm early inflammatory changes of lower airways. The most common change in BAL is the appearance of neutrophils. However, the correlation between neutrophils and the severity and the course of prognosis of the disease is not too good. Inflammatory cells, e.g. neutrophils, have been used to detect inflammatory changes from transtracheal washes or tracheal aspirates.

The respiratory dysfunction in equine COPD can be evaluated by arterial blood gas analysis, requiring expensive equipments or by intrathoracic esophageal pressure difference measurements requiring special instrumentation. Obvious changes in lung function tests, like arterial blood gas- and intrathoracic esophageal pressure-measurements, are detected only if obvious clinical signs are present. Mild diseases are not detected. Lung biopsy has also been used to diagnose equine COPD. However, it can cause severe bleeding. Accordingly, it is not routinely recommended to be used in diagnosing COPD.

Lung function testing before and after administration of atropin has been used to test the reversibility of COPD but atropin causes severe side-effects. Accordingly, it is not recommended for diagnostic purpose (Lavoie, J.-P., In Current Therapy in Equine Medicine, Ed. N. E. Robinson. Saunders Company, Philadelphia, Pennsylvania, USA, pp. 426-437, 1997). The lung function test is used to measure the functional capacities of the whole lung in man and the velocity of the air moving from and to the airways as well as bronchial hyper-reactivity toward graded doses of chemical agents.

Generally, reduced values in lung function testing are due to many different and multiple causes. Bronchoconstriction, for example, narrows the airways due to airway muscular spasms epithelial hypertrophy, sputum obstruction or fibrotic lesions, etc. However, lung function tests neither give any indication of the presence and/or severity of inflammation in the airways nor of an active on-going tissue destructive phase in the respiratory tract disease process. At present, chronic inflammation in the lung is mainly down-regulated by corticosteroid treatment. Most effective, useful and less side-effect causing steroid therapy in modern pulmonology is the use of metered dose inhalers. The aim of the regular use of steroids is to cover and prevent exacerbations of the chronic lung inflammation. Inflammation down-regulating drugs

like corticosteroids, especially inhaled, are recommended both for therapy and prevention of chronic tissue destructive lung disorders, because airway inflammation has shown to be the main and key reason of worsening and progression of these disorders.

As infectious-induced or non-infectiously originated inflammation has grown to be the main target for therapy in acute or chronic respiratory diseases, the need for diagnostic materials and tools to evaluate the presence and severity of said lung inflammations and to identify on-going tissue destructive active phases of lung diseases has increased and there is an acute demand on the field for lung disease prevention, diagnostics and treatment in both human and animals. Peripheral blood has been used for this purpose by measuring circulating mediators, especially extracellular proteins and cytokines. Some mediators have been measured from urine. In general, it is unclear if and how circulating inflammatory mediator levels in serum and urine could specifically differentiate between an active and/or inactive phase of chronic inflammatory lung disease.

Recently, assays to measure some inflammatory mediators such as interleukins, granular proteins, such as extracellular proteins, from hypertonic saline-induced sputum have been introduced. It has also been suggested that measurements of inflammatory cells present in induced sputum as well as cell markers in said inflammatory cells could be useful. Finally, inflammatory mediators such as reactive oxygen species or cytokines ( $H_2O_2$ , leukotrienes, exhaled NO and IL-5) from the condensate of exhaled air are under evaluation.

A correlation between airflow limitations and an imbalance between matrix metalloproteinases (MMP) and tissue inhibitors of matrix metalloproteinases (TIMP) in sputum of patients with asthma and chronic bronchitis has been suggested by Vignola, A.M., et al., (Am. J. Resp. Crit. Med., 158 (6): 1945-1950,

1998). The fact that activated forms of MMP-9 or other MMPs were not shown, seems to indicate a lack of an on-going active and tissue destructive phase in the respiratory tract of their patients. The active and tissue destructive phase of chronic lung disease with enzymatic degradation of lung connective and parenchymal tissue as well as basement membrane, responsible for the various structural and functional alternations, are the most representative characteristics of irreversible changes of the architecture of the lung inflammations with severe functional disabilities and/or dysfunctions. Injuries in lung parenchymal connective tissue and basement membrane is a major complication of the inflammation in lung. Such inflammatory as well as tissue and basement membrane injury contributes for example to the pathological changes in many lung inflammatory diseases, such as bronchiectasis, chronic obstructive pulmonary disease, asthma and other respiratory disorders.

Due to the key role of MMPs and MMP-RMs in tissue and basement membrane destructive processes in various mammalian diseases, considerable effort has been focused on developing MMP-inhibitors in order to inhibit and/or down-regulate the pathologically elevated and activated MMPs at the sites of the active destructive disease process. Less attention has been given to the need of methods and test kits for evaluating and identifying active and tissue destructive phase(s) of lung and respiratory tract disease(s), the severity of inflammatory and tissue destructive activities in the airways as well as for identifying on-going active phase(s) of lung disease processes. Also effects of medication requires efficient test-based follow-up. So far, a successful, rapid and reliable, chair-side method and/or test kit allowing routine determination and evaluation of the severity and active tissue destructive phases of chronic and/or infectious induced acute inflammation in the respiratory tract has not been developed.

The practicing lung physicians, veterinarians and other

persons working in said fields, however, encounter in their everyday work lots of diagnostic problems for which an accurate, rapid, easily available chair- or bed-side method and on-field test kit is urgently needed.

When a patient, is presenting in the consulting room of physicians or veterinarians in hospitals, clinics, private receptions rooms or in animal shelters or under field conditions, it would be desirable to be able to accurately evaluate the severity of inflammation and phase of disease activity in the respiratory system with an unbiased test system giving a picture of the situation in whole lung or part thereof. It would be advantageous to be capable of differentiating a diseased part of the respiratory system from a healthy unaffected respiratory system and especially important to detect early stages of respiratory inflammatory diseases or infections, before the onset of chronic or initial, active phase functional and destructive deficiencies. It would also be desirable to follow up the effects of drug therapy including other kinds of medication or treatment modalities, i.e. to see whether the therapy selected and the dosage of the drug therapy selected is efficient and adequate. An unbiased test result indicating the severity of inflammation also allows a more accurate prediction of the costs of the therapy and especially in veterinary animals the economic feasibility of the therapy to be selected.

Due to increasing environmental pollution, especially air pollution, and the increasing occurrence of allergenicity, there is a great demand for methods and tools for easy monitoring and screening to decide whether natural challenges or other respiratory tract irritating agents in the environment of an individual or individuals cause inflammation in respiratory tracts or to determine if experimental challenge with allergens or other respiratory tract irritating agents causes inflammation in the respiratory tract. Further it would be advantageous to be able to evaluate whether the removal of the

suspected challenging, respiratory tract irritating, inflammatory and infection inducing and/or causative agents eliminate the diseases from the respiratory tracts. In case of infections, allergy or other irritating factors, it is desirable to follow-up the consequences of allergy, i.e. to check whether the elimination of infectious agents, foods or other environmental changes also eliminates inflammatory reactions and responses from the airways and to evaluate whether an atopic individual can tolerate for example a horse, a dog or a bird.

Houses contaminated with mold or other respiratory tract irritating or inflammation inducing agent(s) cause irritation to the inhabitants of the house as well as people working in the house. Possibilities to deduct, whether a house contaminated with mold or other respiratory tract irritating or inflammation inducing agent, is suitable for humans and animals to live in, would be advantageous. It would be especially beneficial, if one could assess the deleterious effects of the contaminants by measuring the reactions of the airways of the inhabitants on the place both before and after sanitation.

For the physicians or veterinarians it is important to be able to determine the suitability of an animal shelter for the animals and also for people working therein. This is especially important in connection with shelters having a small airspace and insufficient ventilation. Methods and tools which could be used in field conditions, would enable the development of standards for respiratory tract irritating or inflammation inducing conditions in animal transporting vehicles and in the facilities used for animals and also to control that the standards are followed. It would also enable evaluation of damage caused by improper conditions.

Nowadays, the physicians or veterinarians often have to decide if an individual or a race horse can be exposed to

physical stress. In such situations, it would be advantageous to have an unbiased more or less scientifically reliable tool for assessing whether the individual can be allowed to participate in physical exercise and to evaluate the health risks connected with participation when the athletes and race or sport animal are presenting with low grade airway inflammation.

The above is an non-exhaustive list of some of the problematic situations frequently encountered in which a guiding confirmatory reply often would be urgently needed and for which an adequate simple solution is still lacking. The lack of methods and reasonably prized, easily available test kits, for making an effective, rapid, on-field, chair- or bed-side assessment for evaluating in the above mentioned situations, is a severe handicap for the medical and veterinary profession.

None of the conventional methods for diagnosing respiratory diseases are fully satisfactory for indicating the risks that an acute inflammation turns to a severe chronic process with consequent tissue injury. The lung function testing does not allow all types and phase(s) of the lung diseases to be detected. Clinical observations are not reliable enough. Radiographic evaluations have to be combined with detailed clinical observations and lung function testing. Morphology studies of the biopsies from respiratory tract are too small and local to give a clear picture of the situation in the whole or part of the lung.

Diagnoses based on breakdown products have not been satisfactory either, because the presence of breakdown products may indicate rapid turnover or synthesis of collagen, not necessarily degradation thereof. Inflammatory mediators have been studied but no sufficiently rapid and specific tests have been designed. Tests based on several host- or bacterial/microbial-derived enzyme activities have been developed, but they are not specific due to broad substrate specificities.



Severe respiratory tract diseases with consequent tissue injuries have been connected with certain cells, such as neutrophils, which are known to produce a variety of inflammatory mediators. Some of these have been suggested as biochemical/immunological markers in the assessment of disease activity.

Assessment of proteolytic enzymes, collagenases, gelatinases and serine proteinases, including matrix metalloproteinases (MMPs) and/or related molecules (MMP-RMs) have been suggested for evaluation of the presence and severity of respiratory tract inflammations. However, due to the broad substrate specificity of the enzymes, none of the suggested methods are capable of measuring one or more MMPs and/or MMP-RMs in a sufficiently accurate, specific and rapid way to provide a reliable tool for diagnosing the severity of respiratory tract inflammation. The enzymatic methods lack the ability to distinguish between different MMP- and/or MMP-RM activities. Therefore, a method specific for one or several MMPs and/or MMP-RMs would be optimal in addressing the course of tissue destruction events in connection with lung disease activity. The present inventors have based on results of Western blottings, shown a correlation between certain collagenases or gelatinases and severity of respiratory tract diseases. After SDS-PAGE and transfer onto nitrocellulose, immunologic staining with labelled polyclonal antibodies the specific enzymes have been identified and correlated with the severity of lung diseases. However, these methods are too laborious and time consuming to be used in routine laboratory work. Moreover, it is unlikely that a rapid chair-side test could be based on electrophoresis.

Accordingly, no acceptable method has so far been developed for assessing the severity of respiratory tract diseases based on a specific measurement of one or more MMPs and or related molecules (MMP-RMs). Although many biochemical methods for measuring collagenase exists, none of them have proved to be

satisfactory for designing a reliable, rapid chair-side test which is a prerequisite for a successful test kit. Immunological methods such as Western blotting, ELISA and other methods discussed above require expensive instruments and facilities. They are tedious and difficult to perform. Thus, they do not provide a reliable, simple and rapid chair-side test.

As addressed above, MMPs and/or MMP-RMs are directly related to the connective tissue destruction and should serve as a specific and sensitive biochemical indicators of progressive respiratory tract disease. Western blot data done with polyclonal anti-MMPs and/or anti-MMP-RMs from respiratory secretion samples suggest that the presence of MMPs and/or MMP-RMs indicate the presence and severity of respiratory tract diseases with minimal false positive and negative results. However, the polyclonal anti-MMPs or -MMP-RMs are not specific enough. Therefore, a test utilizing specific monoclonal antibodies to identify MMPs and/or MMP-RMs would be optimal for determining the lung disease activity and to overcome the problems discussed above. Due to the broad substrate specificities, enzymatic determination does not differentiate between different MMPs and/or MMP-RMs. Thus, it is not feasible to use enzymatic tests. Enzymatic tests are tedious and require laboratory facilities. Even the gelatin test stick developed for diagnosing periodontitis requires a performance time of at least half an hour.

The present invention provides a solution to the problem by providing immunochemical methods and test kits, which are sufficiently specific, rapid and capable of measuring several MMPs and/or MMP-RMs, consecutively or simultaneously, from the same sample and allows an effective, rapid assessment of the inflammatory and disease activity status in respiratory tracts of humans as well as animals. The methods and test kits of the present invention are based on the fact that there is a relation especially between matrix metalloproteinases

(MMPs) and/or matrix metalloproteinase related molecules (MMP-RMs), determined as an increased level of total MMPs and/or MMP-RMs, including both their latent and active forms. Particularly, their activated forms reflect the inflammatory levels, severity of inflammation and especially the active disease phase(s) in respiratory tracts.

Also indicated is the fact that certain MMPs and/or MMP-RMs are more specific than others in assessing certain respiratory diseases and that there is also some differences in specificity and selectivity when diagnosing different respiratory diseases in humans and animals. Thus, it is the objective of the present invention to provide methods and test kits for assessing the cascades of latent and active MMPs and/or MMP-RMs molecules released during respiratory diseases either simultaneously or species by species, selecting the MMPs and/or MMP-RMs, most suited and effective, for the specific diagnostic purpose. Especially, the test is targeted to diagnose the active phase of said disease and/or conditions.

The present invention provides a highly effective diagnostic tool for evaluating the kind of treatment and regimen of therapy needed. The methods and kits of the present invention also provide diagnostic tools for evaluating the severity as well as phase(s) of disease activity of the respiratory tract inflammation. At the same time the methods and test kits provide effective means for follow-up studies and monitoring of the efficacy of the therapies, medications or treatment modalities as well as the dose-treatment response of medication obtained.

Thus, the objectives of the present invention are to provide systems and tools, including methods and test kits for early diagnosis and preventive treatment; to provide systems and tools for following-up the efficacy of treatment and effect of the drug therapies including other medications; to provide

systems and tools especially for easy and rapid chair- or bed-side diagnostics; to diagnose the active tissue destructive phase of said lung disease; to evaluate the timing, dose and regimen of the main treatment modalities and medications; to provide early diagnosis of respiratory inflammation to prevent chronic functional changes; to evaluate the level of inflammation and phases of disease activity in the whole respiratory system or a diseased portion of the respiratory system; to evaluate the health of the unaffected respiratory system or unaffected part of respiratory system; to evaluate which part of the respiratory tissue is affected; to follow up the effects of drug therapies and medications, i.e. to check whether the treatment modality or therapy selected is efficient; to follow up whether the dosage of the drug therapies, medications and treatment modalities selected is efficient; to follow up whether the dosage of the drug selected is adequate; to develop a method to evaluate whether an individual is capable of physical exercise; to develop methods for assessing whether experimental challenge to allergens or other respiratory irritating agents cause inflammation in the respiratory tract; to develop methods for evaluating whether natural challenge in the environment of the individual causes inflammation in respiratory tract; to evaluate whether elimination of the possible challenging agent, which can be of non-infectious or of infectious origin, eliminates inflammation from the respiratory tract; to evaluate suitability of an animal shelter for the respiratory tract of animals or people working therein; to determine whether a house contaminated with mold or other respiratory tract irritating or inflammation inducing agents cause irritation to the inhabitants of the house; to develop a method to test whether elimination of respiratory tract irritating or inflammation inducing agent(s) remove inflammation from the respiratory tract; to evaluate the level of cruelty caused by keeping/having kept the animal in respiratory irritating or inflammation inducing conditions; to develop a tool for physicians and/or veterinarians to evaluate

the ability of a sportsman/sport animal presenting with symptoms of inflammation and/or disease to participate in a race or a game.

But above all, the main objective of the present invention is to provide a immunochemical method and test kit capable of measuring and/or differentiating one or more MMPs and/or MMP-RMs from the same sample with one or more test strips and capable of being used as an easily available, on-field and/or chair- or bed-side tool for diagnosing the severity of respiratory tract disease, the therapy needed, the dose-effect as well as for the differentiation of various diseases by the consulting medical or veterinary professionals in their offices in health centers, clinics and hospitals as well as for on-field veterinary diagnostics. Ultimately, the test could be used for self diagnostics, e.g. by asthmatic patients in contact with their doctors.

### The Summary of the Invention

The characteristics of the method and test kit are as set out in the claims of the present invention.

### A Short Description of the Drawings

Figure 1 shows a diagram of densitometric scanning analysis of Western blot of MMP-9 of induced sputum in patients with bronchial asthma compared to healthy controls.

Figure 2 shows a diagram of densitometric scanning of Western blot of MMP-9 of BAL fluid in patients with different degrees of asthma or treated asthma as compared to healthy controls.

Figure 3 shows a diagram of densitometric scanning of Western blot of MMP-9 of BAL fluid in patients with bronchitis and chronic obstructive pulmonary disease (COPD) as compared to healthy controls.

Figure 4 shows a diagram of densitometric scanning Western blot of MMP-8 of induced sputum in patients with bronchial asthma compared to healthy controls.

Figure 5 shows a diagram of densitometric scanning Western blot of MMP-8 of induced sputum in patients with bronchiectasis compared to healthy controls.

Figure 6 shows a diagram of densitometric scanning Western blot of MMP-8 of BAL fluid in patients with bronchial asthma before and after treatment and bronchiectasis compared to healthy controls.

Figure 7 shows a diagram of densitometric scanning Western blot of MMP-8 of BAL fluid in patients with bronchial asthma before and after treatment and bronchiectasis compared to healthy controls.

Figure 8 shows a diagram of the elevated MMP-8 amounts measured by IFMA-assay utilizing monoclonal antibody specific for MMP-8 were seen in bronchiectasis and asthma patients relative to controls.

Figure 9 shows that positive MMP-8 dip-stick results are strongly associated with elevated MMP-8 IFMA values. Most of MMP-8 dipstick positive subjects have active phase of bronchiectasis or asthma.

Figure 10 shows elevated MMP-8 concentrations ( $\mu\text{g/l}$ ) obtained by IFMA from BAL fluid samples of patients with bronchial asthma, COPD and bronchiectasis in relation to healthy controls.

Figure 11 shows a diagram of densitometric scanning analysis of a Western blot of MMP-13 of induced sputum in patients with bronchial asthma and bronchiectasis compared to healthy con-

trols.

Figure 12 shows a diagram of densitometric scanning analysis of a Western blot of MMP-13 of induced sputum in patients with bronchiectasis compared to healthy controls.

Figure 13 shows a diagram of the results obtained from induced sputum samples analysed with quantitative Western-blotting for MT1-MMP. Elevated MT1-MMP immunoreactivities were found in induced sputum samples from bronchial asthma patients.

Figure 14 shows a diagram presenting results obtained from induced sputum samples analysed with quantitative Western-blotting for NGAL.

Figure 15 shows a diagram presenting gelatinolytic activities of TELF (average  $\pm$ sd) in healthy horses and in horses suffering from COPD. All activities increase significantly. Active products are detected only in the respiratory secretions of COPD horses.

Figure 16 shows a diagram of a densitometric scanning analysis of the amounts of MMP-9 in respiratory epithelial lining fluid (TELF) of healthy horses and horses suffering from COPD as evaluated by scanning the Western blots.

Figure 17 shows a diagram of a densitometric scanning analysis of the amounts of MMP-9 in native tracheal flush fluid of healthy horses and horses suffering from COPD as evaluated by scanning the Western blots.

Figure 18 shows a diagram representing amounts of MMP-9 in a cell-free BAL fluid of healthy horses and horses suffering from COPD.

Figure 19 shows a diagram presenting amount of MMP-8 (average  $\pm$  SD) in respiratory epithelial lining fluid (TELF) of healthy

horses and horses suffering from COPD as evaluated by scanning the Western blots.

Figure 20 shows a diagram presenting amount of MMP-13 (average  $\pm$  SD) in respiratory epithelial lining fluid (TELF) of healthy horses and horses suffering from COPD as evaluated by scanning the Western blots.

## The Detailed Description of the Invention

### Definitions

In the present invention the terms used have the meaning they generally have in the fields of human and veterinary medicine and diagnostics, especially in chair- or bed-side diagnostics as well as immunochemistry. Some terms, however, are used with a somewhat deviating or broader meaning in this context. Accordingly, in order to avoid uncertainty caused by terms with unclear meaning some of the terms used in this specification and in the claims are defined in more detail below.

The term "respiratory tract inflammation" means the various alternations, which are characteristic of severe lung disease activity and the initiation of lung tissue destruction and/or of diseases leading into irreversibly diseased lung, often related to both severe structural and functional abnormalities, but the term also incorporates inflammation of the mucous membrane of the nose (rhinitis), including allergic or infectious rhinitis. In the present invention it has been shown that an elevated or high level of MMPs and MMP-RMs correlates with the severity of the disease and indicates to the physician or veterinarian that special measures are required. Damages to, and tissue destruction of, lung parenchymal connective tissue and basement membranes, is a major complication of the inflammatory response, which can be induced or caused by allergenic challenge or other irritative or allergy inducing agent, microbial, bacterial, viral, chlamy-



dial, mycoplasmal, protozoal, fungal and/or parasitic infections alone or in any possible combinations without being limited to the inductive or infectious agents. Such infections as well as inflammatory lung tissue and basement membrane injuries contribute, for example, to the pathological changes in many lung inflammatory diseases, such as bronchiectasis, cystic fibrosis, chronic obstructive pulmonary disease, allergic, microorganism caused, asthma and respiratory disorders, syndromes, emphysema and HIV-positive-lung diseases. They are correlated to enzymatic degradation of lung connective and parenchymal tissue and basement membrane. The tissue destruction of lung extracellular matrices and basement membrane reflects active phase of said lung diseases.

The term "respiratory tract inflammation" in this context includes, in its broadest aspect, not only diseases in the lungs but also inflammations in nose tract membranes. The term means an on-going active inflammatory condition in the respiratory tract related with a high risk of respiratory tract injury. Said condition can be correlated to an elevated amount or level of total MMPs and/or MMP-RMs or a high degree of activation of said molecules, their concentration being above the concentration found in respiratory secretion samples from healthy persons.

The term "diagnosing" means judging, predicting, assessing or evaluating from the recorded results, the severity and phase(s) of disease activity of inflammation in respiratory tracts, based on determination, measurement or detection of MMPs and/or MMP-RMs with the test kits or methods of the present invention. Evaluation of the efficacy of medical treatment, predicting the risk for progress of said diseases as well as predicting the risk of physical stress with said methods and kits is also incorporated into the term.

The term "respiratory secretion sample (RS)" means a sample collected from upper or lower parts of the respiratory tract

representing the whole lung or part thereof. In other words, it gives a more representative picture of the situation in the whole lung than a biopsy obtained from a specific tiny area of the lung. The term "sample collecting" in the present invention means methods with which a respiratory or a nasal secretion sample is obtainable. Respiratory secretions (RS) typically contain topical mucoid and fluid substances from the epithelial surface of trachea, bronchi, bronchioli and alveoli and they are also obtainable by transtracheal washes or tracheal aspirates.

Representative respiratory samples are, for example, obtained from lower tracheal level, including tracheal epithelial lining fluid (TELF), by using standard amounts of fluid, e.g. saline, for flushing. The so called tracheal flush fluid (TFF) represents both the amount of the substance in TELF and the amount of TELF in lower tracheal level. Typical representatives of respiratory samples are coughing products, such as sputum (S) or induced sputum (IS), which are obtainable by a standardized method by inhalation of hypertonic saline, followed by coughing and collecting the respiratory sample with a standardized method. Another example of respiratory samples is bronchoalveolar lavage fluid (BALF), a respiratory sample obtainable from bronchoalveolar level, using a flushing fluid (physiological saline) with a standardized procedure, where an equal, corresponding area of respiratory tract surface is flushed with a standard volume with a standard procedure. In animals, particularly in horses, the most preferred collection method is obtaining tracheal epithelial lining fluid (TELF) by flushing with sterile saline.

The term "matrix metalloproteinases (MMPs, matrixins)" in normal context means the family of 20 or more genetically distinct, but structurally related matrix metalloproteinases, capable of degrading almost all extracellular matrix (ECM) and basement membrane (BM) components, serine proteinase

inhibitors (such as serpins and plasminogen activator inhibitors), cytokines, cell surface components, adhesion molecules and complement components. The number of MMP-family members and/or MMP-RMs is increasing all the time as new members are detected. This invention covers by the terms "MMPs" and "MMP-RMs" all present and future new MMP-family members or MMP-RMs. Said Zn-binding metallo-endopeptidase enzymes are unified as a family of proteinases with significant structural homologies and similar, but not identical enzyme properties. In addition to their genetically distinct, but structurally related primary structure, which allows their classification into series of sub-types and -groups, said proteinases are characterized by their widely divergent substrate specificities and by virtue of their susceptibility to activation by proteolytic or non-proteolytic removal of the N-terminal propeptide from their latent proforms. MMPs can also be activated without any changes in molecular size (Handbook of Proteolytic Enzymes, Eds, Barrett, A.J. Rawlings, N.D. & Wessner, J.F., pp. 1-1696, Academic Press, 1998; Shapiro, S.D., Curr. Opin. Cell Biol., 10(5):, 602-608, 1998).

The term "membrane-type matrix metalloproteinases (MT-MMP)" means a recently discovered matrix metalloproteinase- or MMP-subfamily, substantially distinct from the conventional or extracellular MMPs. MT-MMPs differ from other MMPs in a number of ways, of which the major part is explained by their trans-membrane domain related to their cell-surface binding (Handbook of Proteolytic Enzymes, Eds, Barrett, A.J. Rawlings, N.D. & Wessner, J.F., pp. 1-1696, Academic Press, 1998; Shapiro, S.D., Curr. Opin. Cell Biol., 10(5):, 602-608, 1998). MT-MMPs contain 24 amino acid hydrophobic domains, which contribute to the attachment, i.e. the binding to cell membrane. MT-MMPs can attack collagens and gelatin, but MT-MMPs are additionally important in cell surface associated activation, especially of proMMP-2.

The term "matrix metalloproteinase related molecules (MMP-RMs)" also includes molecules which are present in close connection with MMPs, such as neutrophil gelatinase associated lipocalin (NGAL), which forms an approximately 120 kD complexes, comprising a 25 kD lipocalin associated with 92 kD gelatinase B (MMP-9).

The terms "MMPs and/or MMP-RMs" cover all possible splice variants of MMPs and MMP-RMs. Such splice variants have been identified and characterized for MMP-8 (Hu, S-H., et al., FEBS Letters, 443: 8-10, 1998). Due to the lack of a secretory signal sequence, the splice variants of MMP-8, as well as of other MMPs and MMP-RMs, are probably not secreted proteins. Nevertheless, they can be released into body fluids such as respiratory tract secretions during cell death associated with lung tissue destructive diseases. The term "MMP-RMs" further cover key MMP-regulatory proteins such as their serine proteinase activators such as elastase and trypsins (Sorsa, T., et al., J. Biol. Chem., 272(34): 21067-21074, 1997). In the diagnostic tests described in the present invention these molecules can be used alone and/or in all possible combinations.

In addition to activation by other proteinases (serine proteinases, bacterial proteinases and other MMPs), latent proMMPs can also be activated by non-proteolytic means. Especially, oxidants or reactive oxygen species are important in vivo in inflammatory diseases (Saari, H., et al., Biochem. Biophys. Res. Commun., 171(3), 979-987, 1990). ProMMPs can also be autoactivated (Sorsa, T., et al., J. Biol. Chem., 272(34): 21067-21074, 1997; Westerlund, U., et al., J. Dent. Res., 75(8): 1553-1563, 1996; Handbook of Proteolytic Enzymes, Eds, Barrett, A.J., Rawlings, N.D. & Wessner, J.F, pp. 1-1696, Academic Press, 1998; Shapiro, S.D., Curr. Opin. Cell Biol., 10(5): 602-608, 1998). In order to identify the factors capable of initially activating the proMMP-activation cascade, it has been shown that trypsin-2 can induce proMMP-activation

and proMMPs activating in the activation cascade (Sorsa, T., et al., J. Biol. Chem., 272 (34): 21067-21074, 1997).

As a conclusion "matrix metalloproteinases" in its broadest aspect in the present invention, covers not only normal extracellularly released "matrix metalloproteinases (MMPs)" but also "membrane bound matrix metalloproteinases (MT-MMPs)" and "matrix metalloproteinase related molecules (MMP-RMs)" both in their latent and active forms as well as substances capable of activating and inhibiting said MMPs, MT-MMPs and MMP-RMs. All said forms are in the present invention covered under the term "matrix metalloproteinase associated and/or related molecules (MMP-RMs)".

In the most preferred embodiment of the present invention the term "matrix metalloproteinases and/or related molecules" comprises above all MMP-2, MMP-8, MMP-9, MMP-13, MMP-14 (MT1-MMP) and/or NGAL, particularly in their activated forms.

The term "matrix metalloproteinase (MMPs) and/or related molecules (MMP-RMs)" comprise not only the above defined molecules, including their isoforms of different origin, either as separate entities or in any combinations. The term covers all listed MMP-RMs, either in their latent or active forms or in any combinations of said forms as well as fragmented, truncated, derivatized and/or complexed forms thereof as well as splice variants.

The term "isoform" refers to the different forms of the same protein, which originate from different sources, e.g. different species of animals. In the present invention the term includes fragments, complexes and their derivatives. For example, active collagenases, such as metalloproteinase MMP-8, are generated by the cleavage of the proenzyme to form an active enzyme. Different reactions, including different enzymatic and non-enzymatic reactions, proteolytic and non-proteolytic, are capable of creating an active enzyme.

These varied reactions cleave the proenzyme differently, generating different molecular species also included within the term "isoform". MMPs can, however, be activated and autoactivate without any changes in their molecular sizes.

It has been shown that some MMPs and/or MMP-RMs correlate better than others with the severity of the respiratory disease and also that certain MMPs and/or MMP-RMs correlate better when applied to humans than to animals. Thus, it is convenient to select from the MMP and/or MMP-RM-group, the member(s) correlating best to the selected diagnostic purpose in order to provide more selective and/or specific embodiments of the present invention. For example, the present invention shows that MMP-8 correlates very well with the severity of COPD and bronchiectasis. MT1-MMP correlates especially with the severity of bronchial asthma in man. MMP-9, especially in its active forms or as fragments, reflecting activation, correlates well with the severity COPD in horses.

The amount of MMPs and/or MMP-RMs in respiratory tract fluids are determined with "binding substances" including binding proteins or peptides as well as antibodies, which recognizes matrix metalloproteinases (aMMPs) and/or related molecules (aMMP-RMs). The term "binding substance, which recognizes matrix metalloproteinases and/or related molecules (aMMPs and/or aMMP-RMs)" in its broadest aspect means any substances capable of specifically recognizing and binding at least one or more or a part of one or more MMP-RMs of the present invention. Such substances are, for example, receptors or binding proteins or peptides, capable of specifically binding MMPs and/or MMP-RMs, but above all they mean antibodies capable of specifically recognizing one or more MMP and/or MMP-RMs alone or in any combination. The antibodies include both polyclonal and/or monoclonal antibodies as well as fragments or derivatives thereof. The prerequisite being that the binding substances specifically recognize at least one of the above-defined MMPs and/or MMP-RMs or parts, e.g. active sites

or epitopes thereof, either alone or in any combination.

Said "aMMPs" and "aMMP-RMs" can be produced using any MMP and/or MMP-RMs, their isoforms as well as their fragments, derivatives and complexes which are capable of acting as "antigens". Thus, the terms "aMMPs and/or aMMP-RMs" refers to any compositions or materials, particularly antibodies elicited as a specific response to said MMP- and/or MMP-RM-composition or -material. Said antibodies are producible by conventional techniques for producing polyclonal antibodies as well as monoclonal antibodies. The methods for preparing monoclonal antibodies include hybridoma techniques. Fragments of antibodies or other binding proteins like specific binding peptides can be developed by phage display techniques and produced by recombinant DNA techniques. All methods are well known by those skilled in the art and described in laboratory handbooks.

The term "immunoassay" refers to a method or procedure capable of detecting and/or measuring a substance such as MMPs or MMP-RMs wherein the active and specific reagents include at least one antibody capable of specifically binding said substance. Well known examples of immunoassays are radioimmunoassays (RIA), radioimmunometric assays (IRMA), fluoroimmunometric assays (IFMA) enzyme immunoassays (EIA), enzyme-linked immunosorbent assays (ELISA), fluoroimmunoassays (FIA), luminescence immunoassays, immunoagglutination assays, turbidimetric immunoassays, nephelometric immunoassays, etc. All methods are well known by those skilled in the art and described in laboratory handbooks. Basic types of immunoassays include above all "Sandwich assays", which are defined below.

The term "Sandwich Assay" refers to an immunoassay using at least two antibodies capable of detecting or quantifying the amount of antibody or antigen in a sample. In the assay two different antibodies capable of binding two different, non-overlapping (non-competitive) epitopes on an antigen are

used. Different types of "Sandwich Assays" exist as described below.

The term "Lateral Flow Technique" refers to an immunoassay using immunochromatographic principles. It is typical for the test that the sample or test solution, which is in liquid form moves along a test strip in contrast to the "Flow-Through Technique" in which the test solution is allowed to flow through a membrane in a test device.

The term "Flow Through Technique" refers to an immunoassay often based on the sandwich technique. The antigen containing sample or test solution is applied as a spot and is allowed to diffuse through a membrane in a device.

"Chair- or bed-side as well as on-field assays" refer to tests or procedures performed without any laboratory facilities and without the need of qualified laboratory personnel. "Chair-side tests" can be made by the physician, while the patients are visiting the doctors, whereas "bed-side assays" can be made while the doctors are on their daily bed-side visits to the patients. "On-field assays" mean tests made by a veterinarian under field conditions for example in animal shelters. Also included are so called "self-tests", where the tests can be carried out by the patients themselves in close cooperation with the doctor. "Chair- or bed-side assays and on-field assays" are preferably performed on "solid carriers" like test strips. Various MMPs and/or MMP-RMs can be detected simultaneously on the same stick.

#### The General Description of the Invention

Conventionally, lung function test have been used to measure the functional capacities of the lung and the velocity of air from and to airways as well as bronchial hyperreactivity against graded doses of chemical agents. In early obstructive respiratory disease such as bronchial asthma and chronic



obstructive pulmonary disease (COPD), inflammation precedes lung function alterations. Reduced values in lung function testing can be due to bronchoconstriction, the narrowing of the airways due to sputum obstruction and/or epithelial hypertrophy, but can also result from fibrotic lesions as a mark of chronic changes in the airways. However, lung function tests show only indirect evidence of worsening lung function, giving no direct evaluation of the cause, stage and severity of chronic inflammatory process. It does not measure the presence or level of inflammation in the airways.

Prognosing the status and stage of active on-going inflammation in chronically ill patients has always been a goal of physicians and veterinarians. Early diagnosis of the onset of chronic inflammation of infectious or non-infectious origin in the airways gives possibilities to prevent the onset or worsening of obstructive syndromes associated with active inflammation. At the same time, the increased incidence of allergenicity, seen as disorders in the respiratory tracts and correlating with increased air pollution, has created a demand for methods and tools for evaluating health risks connected with disorders in the respiratory tract.

It is known that matrix metalloproteinases (MMPs) take part in the degradation of extracellular matrix (ECM), epithelium, endothelium and basement membrane (BM) by cleaving almost all of their protein constituents (Handbook of Proteolytic Enzymes. Eds Barrett, A.J., et al., pp. 1-1696, Acad.Press., 1998). The studies of the involvement of MMPs in the pathogenesis of respiratory diseases are concentrated to the pathogenetic aspects of MMPs. The use of MMPs in clinical diagnostics of active phases of lung diseases, however, has remained completely uninvestigated. Besides taking part in remodeling in physiological conditions, pathologically elevated levels of MMPs may cause tissue destruction during pulmonary inflammatory conditions like emphysema, bronchiectasis, asthma and chronic obstructive pulmonary disease (COPD), HIV-infection related lung diseases

respiratory diseases as well as cystic fibrosis. In all fluid samples, such as diseased lavage fluid, sputum, etc., it has been shown that when MMPs are activated, the actual disease process is in the active phase in respect to lung tissue destruction.

In horses, gelatinolytic and collagenolytic MMP-activities, but not immunoreactivities, have been shown to be increased and activated in the tracheal epithelial lining fluid (TELF) suffering from COPD. Due to the broad substrate specificities of these gelatinolytic and collagenolytic activities, it has not been possible to identify what gelatinolytic and collagenolytic activities are raised in severe respiratory tract inflammations and it has not been possible to develop any accurate, rapid and specific chair-side tools based on the observations.

Elevated amounts of MMPs and/or MMP-RMs alone or in any combinations reflect the presence of inflammation with or without infectious origin, but only a pathologically increased degree of activation of MMPs is a relevant proof of an active phase, actually on-going tissue destructive disease process at the site of inflammation. Even if protein synthesis and release of extracellular and cell-surface bound MMP as well as inhibition by body's major MMP-inhibitors, such as  $\alpha$ -2-macroglobulin and tissue inhibitors of MMPs (TIMPs) effect the level of MMPs, it is the activation of the latent proforms of matrix metalloproteinases (proMMPs) alone and/or MMP-RMs in any combinations that is the key regulation step in respect to on-going lung tissue destruction and its active phase.

It has been found that the activity of MMPs and/or MMP-RMs typically increase mainly at the site of inflammation but an increase in the general circulation MMP-levels have not been detected in connection with respiratory tract inflammatory processes, indicating the importance of using respiratory tract samples. Therefore, the MMPs have to be measured from the tis-

sue fluids, such are epithelial lining fluid (ELF), broncho-alveolar lavage fluid (BALF), induced sputum (IS) and sputum, which represents the whole lung or part of the lung.

MMP- and/or MMP-RM-levels in respiratory secretion samples representing whole lung or part of the lung have been assessed by various known methods. The inventors have estimated the levels of latent and active MMP and/or MMP-RMs in respiratory secretion samples representing whole lung as well as sectional lung samples from healthy and sick humans and animals, by Zymographic methods, IFMA and Western blots using specific antibodies.

Different MMPs and/or MMP-RMs have also been identified by Western blotting. After SDS-PAGE of the enzyme preparations, the bands have been transferred onto nitrocellulose and characterized by staining with polyclonal antibodies specific to said MMPs and/or MMPs/MMP-RMs (Lauhio, A., et al., Clin Exp Immunol 1994, 98: 21-28; Sorsa, T., et al., Ann. N.Y. Acad. Sci., 732:112-131, 1994; Westerlund, U., et al., J. Dent. Res., 75 (8): 1553-1563, 1996). With methods like this, specific results indicating correlation of severity of disease and degree of activation of pathologically elevated levels of MMPs and/or MMP-RMs have been obtained, but said methods are far too laborious and time consuming to be used routinely. Moreover, it is not feasible at present to develop a rapid chair-side test based on any kind of electrophoresis. However, a severe respiratory inflammation should be diagnosed and molecularly identified by the veterinarian in the animal shelter. Therefore, the diagnosis should be aimed at evaluating the inflammatory grade, and its severity and active phase of disease from respiratory sample, representing lung tissue destruction in order to be able to give appropriate treatment regimen at the right time, as well as to monitor the effect of anti-inflammatory, -tissue destructive and -collagenolytic therapy. Besides measuring the level of inflammation, elevated MMP- and/or MMP-RM-levels should give

information of the degree of tissue destruction associated with the disease severity in the lung, the outcome of therapy including treatment modalities as well as the prognosis of the disease. A higher level of MMPs and/or MMP-RMs, which means a greater level of tissue destruction and the need of a more guarded prognosis, selection of beneficial treatment modalities and medical treatment as shown in the present invention. Specific therapy regimens are related to disease severity and actual activity.

Proteases released by resident lung connective tissue cells (fibroblasts) and airway lining epithelia and glandular cells as well as infiltrating inflammatory cells (neutrophils monocytes, macrophages and plasma cells) have been suggested to play a major role in lung tissue and basement membrane injury and destruction associated with active inflammatory response. At least two classes of proteases have been linked to inflammatory lung tissue injury, i.e. matrix metalloproteinases (MMPs, matrixins) and serine proteinases (elastases, trypsins, chymotrypsins, etc.). Said enzyme groups exert differing, but also overlapping as well as supplementing substrate specificities and modes of destructive action and are suggested to form cascades in which serine proteinases initially activate latent proMMPs (Sorsa, T., et al., J. Biol. Chem., 272 (34): 21067-21074, 1997) to be catalytically competent at the sites of active phase of inflammation *in vivo*.

Based on the fact that the amount and degree of activation leading to elevated catalytic activity of MMPs and/or MMP-RMs increase mainly at the site of inflammation whereas an increase of the general circulating matrix metalloproteinase levels cannot be detected in respiratory inflammatory processes, the inventors developed methods and test kits by the aid of which the MMPs and/or MMP-RMs can be measured from respiratory secretion samples, which represent the situation in the whole lung or part of the lung. Such fluid samples are epithelial lining fluid (ELF), bronchoalveolar lavage fluid

formation. All said MMPs and/or MMP-RMs are incorporated into the scope of the present invention.

Said MMPs and/or MMP-RMs are essential to the ability of cells to invade and migrate through membranes and tissue barriers of various bodies, but additionally, they are characterized by a direct degradative action on extracellular membrane (ECM) and basement membrane (BM) protein components. Additionally, MMPs degrade and inactivate serine proteinase inhibitors (serpins), adhesion molecules, cell surface components and cytokines as well as pro-inflammatory mediators and complement components. Thus, MMPs are expressed and synthesized by body cells and tissues in normal, remodelling, turnover situations at low levels, but in abnormal situations, e.g. in connection with inflammation diseased lung tissue, the MMPs and/or MMP-RMs are expressed at pathologically elevated levels often associated with increased MMP and/or MMP-RM activation that can occur and act in cascades, overcoming the body's own endogenous anti-proteinase shield. The last mentioned characteristic is especially demonstrated and utilized in the present invention, in which the elevated expression levels of various MMPs and/or MMP-RMs usually is associated with an increased degree of their activation in diseased respiratory secretion samples, whereas low levels of MMPs and/or MMP-RMs in catalytically incompetent latent proforms are present constantly. In particular MMPs and/or MMP-RMs are distinguished from other proteinases by virtue of their susceptibility to activation by proteolytic removal of N-terminal propeptide from their latent proforms. In addition to activation, the proMMPs, i.e. the latent proforms, are key steps in their extracellular regulation. MMPs are further regulated at transcriptional level, protein synthesis and release levels. Especially, the complexed or derivatized MMP-forms (MMP-RMs), trapped or bound by endogenous inhibitors, i.e., tissue inhibitors of MMPs (TIMPs) and  $\alpha$ -2-macroglobulin ( $\alpha$ -2M) represents *in vivo* activated MMPs and/or MMP-RMs at the site of disease (Handbook of Proteolytic Enzymes, Eds., Barrett

(BALF), induced sputum (IS) and sputum. The inventors were able to show that the sputum, induced sputum or bronchoalveolar lavage fluid (BAL) could be used to represent the situation in the airways at the moment of obtainment of the material. Using these respiratory secretion materials for testing MMPs and/or MMP-RMs, the inventors could show that it is possible to predict severe worsening of airway limiting diseases and to follow the course of the exacerbation and the results of applied treatment.

As defined above the MMPs have widely divergent substrate specificities and activities, which in addition to their structure, allow their classification into series of sub-types. The collagenases include interstitial collagenase-1 (MMP-1), neutrophil collagenases or collagenase-2 (MMP-8) and collagenase 3 (MMP-13), characterized by their ability to specifically degrade triple helical regions of interstitial collagens I, II and III. The gelatinases include gelatinase A and B (72 kD type IV collagenase [MMP-2]) and 92 kD type IV collagenase [MMP-9], respectively), characterized by their ability to specifically cleave gelatins and native type IV collagen, elastin and other substrates. Stromelysins (MMP-3, MMP-10 and MMP-11) characteristically degrade a variety of substrates including aggrecan, fibronectin, laminin, numerous collagen types and play a role in activation of other latent proMMPs. The MMP-family includes several other MMPs that do not fit into the collagenase, gelatinase and stromelysin sub-families. Matrilysin (MMP-7; PUMP-1) differs substantially in structure lacking the C-terminal domain common to other MMP-family members. Metalloelastase (MMP-12) is characterized by its ability to degrade elastin. MMP-20 (enamelysin) can degrade amelogenin and is shown to be involved in tooth development (Llano, E., et al., Biochemistry, 36 (49):15101-08, 1997) but also in cancer progression (Salo, T., et al., J. Dent. Res., 77:879, Abstr 1978, 1997). Previously, membrane-type MMPs were thought to be solely expressed on cell surfaces and being especially associated with cancer and metastasis

A.J., et al., pp. 1-1696, Academic Press, 1998).

So far it has not been clarified, whether different forms of MT-MMPs are expressed and released in extracellular inflammatory body fluids. Results obtained and described for the first time in the present invention, indicate that elevated levels of soluble MT1-MMP in active/activated forms(s) are present in induced sputum/BAL fluid of lung diseases like human bronchial asthma with active MT1-MMP forms occurring only in diseased sputum. Also MMP-2 was activated in the same samples suggesting potential involvement of this MT1-MMP-MMP-2 cascade in lung diseases like human bronchial asthma.

The active phase(s) of lung disease, lung inflammation and/or lung tissue destruction are evaluated by recording MMPs and/or MMP-RMs, such as MMP-2, MMP-8, MMP-9, MMP-13, MMP-14 and NGAL. MMP-RM immunoreactivities are measured from respiratory samples (RS), TELF (Tracheobronchial epithelial lining fluid), BALF (Bronchoalveolar lavage fluid), S (Sputum) and IS (Induced sputum). Tracheobronchial epithelial lining fluid (TELF) is sucked through a catheter inserted through the biopsy channel of an endoscope. Tracheobronchial flush fluid can also be obtained by flushing with NaCl through a catheter inserted through the biopsy channel of an endoscope or as tracheobronchial flush fluid obtained by transtracheal wash (Roberts, C.A., Equine Vet. Educ., 4: 266-268, 1992). Bronchoalveolar lavage fluid (BALF) is obtained by the use of a fiber-endoscope (Baugham, R.P., Bronchoalveolar Lavage, Mosby Year Book, Inc., 1992) and induced sputum by inhalation of hypertonic NaCl (Kips, J.C., et al., Eur. Respir. J., Suppl., 26:9s-12s, 1998; Kips, J.C., et al., Eur. Respir. J., 11 (3): 529-533, 1998). Air condensate of the respiratory tract exhalation air is obtained accordingly.

Thus, in the present invention, it has for the first time been shown that, in addition to matrix metalloproteinases

synthesized and subsequently released to extracellular milieu, also membrane-type matrix metalloproteinases (MT-MMPs), which previously were believed to be solely bound to cell surfaces and/or cell membranes can, in fact, be found in bronchoalveolar lavage fluid/ induced sputum supernatant of human bronchial asthma and bronchiectasis patients. Said elevated levels of MT-MMPs are also activated in the diseased fluid, whereas the lower levels found in healthy fluid cells remain inactive. Thus, as clearly evidenced by the present invention elevated and activated levels of membrane type matrix metalloproteinases (MT-MMPs) in induced sputum/BAL fluid of human bronchial asthma patients and in lung fluid samples, can similarly to other MMPs and/or MMP-RMs, also be used alone or in any combinations in diagnostic tests.

Corresponding observations were made in connection with a representative of the so called "matrix metalloproteinase related molecules", i.e. neutrophil gelatinase associated lipocalin (NGAL), which form an about 120 kD complex comprising a 25 kD lipocalin associated with 92 kD gelatinase B (MMP-9). Elevated levels of monomeric, multimeric and/or fragmented NGAL immunoreactivity in relation to healthy controls were observed in induced sputum supernatant fluid of bronchial asthma and bronchiectasis patients. Thus, MMP-associated and/or regulatory molecules (MMP-RMs), such as NGAL, can be used in the diagnostic chair-side test devices of the present invention.

The antibodies raised against matrix metalloproteinases (MMPs) and/or related molecules (MMP-RMs) and being capable of specifically recognizing them, were used by the present inventors in immunoblot analysis of respiratory secretion samples obtained from human beings and horses. The results indicated clearly that each genetically distinct extracellular- and membrane-type member of the MMPs and/or MMP-RMs not only their active and/or latent forms, splice variants, but also their derivatized, complexed, fragmented, trapped and/or truncated



forms, reflect activated MMPs bound and/or trapped to their inhibitors and other regulatory proteins, such as  $\alpha$ -2-macroglobulin ( $\alpha$ -2M), tissue inhibitors at MMPs (TIMPs) and NGAL. Derivated, fragmented and/or truncated forms as well as splice variants of extracellular and membrane-type MMPs and related molecules also reflect MMP-activation and/or MMP-activation cascade caused by severe inflammation and tissue destruction in respiratory tract. Release and the presence of intracellular splice variant products in body fluids such as RS-samples can reflect cell death at sites of inflammation/disease in lungs.

Various representatives of poly- and monoclonal antibodies recognizing MMPs and/or MMP-RMs listed below are can be produced by per se known methods, but they are also commercially available for example from the following sources.

#### Monoclonal antibodies

MMP-1: (A.F. Schuetzdeller Biochemicals; Triple Point Biologics; Research & Diagnostic Systems Inc.; Fuji Chemicals Limited; Oncogene Research Products; Calbiochem-Novabiochem Corp.; ICN Biomedicals); MMP-2: (A.F. Schuetzdeller Biochemicals; Triple Point Biologics; Research & Diagnostic Systems Inc.; Fuji Chemicals Limited; Oncogene Research Products; Calbiochem-Novabiochem Corp.; ICN Biomedicals); MMP-3: (A.F. Schuetzdeller Biochemicals; Triple Point Biologics; Research & Diagnostic Systems Inc.; Fuji Chemicals Limited; Oncogene Research Products; Calbiochem-Novabiochem Corp.; ICN Biomedicals); MMP-7: (A.F. Schuetzdeller Biochemicals; Research & Diagnostic Systems Inc.; Fuji Chemicals Limited; Oncogene Research Products; MMP-8: (A.F. Schuetzdeller Biochemicals; Fuji Chemicals Limited; Calbiochem-Novabiochem Corp.; Oy Medix Biochemica Ab; Oncogene Research Products); MMP-9: (A.F. Schuetzdeller Biochemicals; Triple Point Biologics; Research & Diagnostic Systems Inc.; Fuji Chemicals Limited; Oncogene Research Products; Calbiochem-Novabiochem Corp.; ICN Biomedicals); MMP-10: (Fuji Chemicals Limited); MMP-12: (Re-

search & Diagnostic Systems Inc.) MMP-13: (Fuji Chemicals Limited; Oncogene Research Products); MT1-MMP: (A.F. Schuetzdeller Biochemicals; Fuji Chemicals Limited; Oncogene Research Products; Calbiochem-Novabiochem Corp.); MT2-MMP (Oncogene Research Products) and MT3-MMP: (Fuji Chemicals Limited).

#### Polyclonal antibodies

MMP-1: (Biogenesis Ltd; Chemicon International Inc.; Triple Point Biologics); MMP-2: (Triple Point Biologics; Chemicon International Inc.; Biogenesis Ltd.); MMP-3: (Triple Point Biologics; Chemicon International Inc.; Biogenesis Ltd.); MMP-7: (Triple Point Biologics); MMP-8: (Triple Point Biologics); MMP-9: (Biogenesis Ltd.; Chemicon International Inc.; Triple Point Biologics); MMP-11: (Triple Point Biologics); MMP-12: (Triple Point Biologics); MMP-13: (Triple Point Biologics); MMP-18: (Calbiochem-Novabiochem Corp.); MMP-19: (Triple Point Biologics; Calbiochem-Novabiochem Corp.); MT-1 MMP: (Chemicon International Inc.; Triple Point Biologics; Oncogene Research Products); MT-2 MMP (Calbiochem-Novabiochem Corp.; Triple Point Biologics); MT-3 MMP: (Calbiochem-Novabiochem Corp.; Triple Point Biologics); MT-4 MMP: (Calbiochem-Novabiochem Corp.); and pro-MMPs (Biogenesis Ltd.).

Monoclonal antibodies of the present invention have been developed according to the original technique of Köhler and Milstein (Nature 256, 495, 1975). Methods for producing said antibodies recognizing MMP-8, especially in its active form are described in the patent US 5,736,341, which is herewith incorporated by reference. Similarly, monoclonal antibodies recognizing other matrix metalloproteinase related molecules can be produced by those skilled in the art. As representatives of binding substances recognizing MMP-RMs, those binding substances recognizing NGAL can be mentioned. Methods for their detection and production are described in the patent US 5,866,432, which is also hereby incorporated by reference.

The antibodies can optionally be tagged with a label or marker molecule capable of making the presence or absence of the MMPs and/or MMP-RMs alone or in combination recordable. Various labels, markers or tags, also called tracer when combined with the antibodies or respective antigens, are known and described in literature, laboratory handbooks and patent publications. Such labels or markers are, for example, coloured latex particles, fluorochromes, liposomes, metal colloids, etc. However, it is to be noted that it is not necessary to use such labels and markers. In turbidimetric assays for example, only one polyclonal antibody is used. When binding to the antigen the sample solution gets turbidic. The turbidity is caused by antibody-antigen-aggregates forming during the reaction. Said aggregates can be detected visually.

Based on the results obtained and the antibodies available the present inventors developed new methods and test kits for an effective, rapid, and reliable assessment of the inflammatory status and tissue destruction status as well as to identify the phases of disease activity in respiratory tracts of human beings and animals. The methods and test kits of the present invention are based on the fact that there is a relation especially between MMP-activation, as measured e.g. from total MMPs, such as MMP-2, MMP-8, MMP-9, MMP-13, MMP-14, MT1-MMP and NGAL, especially their activated forms, and the severity of inflammation, tissue destruction and phase(s) of disease activity in respiratory tracts. Cell death at the sites of lung disease can be measured by analysing MMP- and/or MMP-RM splice variants. Also indicated is the fact that certain MMPs and/or MMP-RMs alone or in any combination are more specific than others in assessing a certain lung disease and that there is some differences in specificity and selectivity for man and animals. Hence, it is advantageous to develop kits by which a multitude of MMPs and MMP-RMs alone or in combination could be determined simultaneously, either on the same test strip or on separate test strips. In preferred embodiments the MMPs and/or

MMP-RMs, which were shown to be most suitable or effective for a specific diagnostic purpose, were selected for the test kit, either alone or in any combination.

Different numeric results obtained in the experiments reflect the use of different sets of binding substances or antibodies as well as other variation in the test conditions. However, it is important to notice that for the active MMPs and/or MMP-RMs the ratio between diseased and healthy persons remains approximately the same and a qualitative, semiquantitative or even quantitative test can be developed for chair- or bed-side assessment of the severity of the diseases in the respiratory tract. The results can be recorded visually or by an instrument either directly or indirectly by adding a substrate capable of making the binding reaction recordable.

These findings indicate that a immunochromatographic test using monoclonal antibodies which measures either total, i.e. both latent and active MMPs or MMP-RMs alone or in any combination is fully sufficient for diagnosing respiratory disease activity. The invention disclosed in the present patent specification provides a highly effective diagnostic tool for an accurate evaluation of the kind of treatment and regimen of therapy needed. The method and kits of the present invention also provide alternative chair- or bed-side and on-field diagnostic tools for evaluating the severity of the respiratory inflammation and the risks caused by infections and/or physical stress in connection with said diseases. At the same time the method and test kit provides an effective tool for follow up studies of the efficacy of the therapy or treatment as well as the dose-treatment response obtained.

Based on these founding methods and test kits for diagnosing the level, and/or severity of inflammation as well as the active phase of lung tissue destructive disease processes in the respiratory tracts, evaluating the efficacy of drug treatments, other treatment modalities, other medications

and/or predicting the risk for progress of said diseases, wherein the detection is performed as a rapid and reliable immunological chair-side assay using respiratory secretion samples by specifically measuring one or more MMPs and/or MMP-RMs, even membrane-type-MMPs (MT-MMPs), MMP activators, (included in MMP-RMs) and MMP- or MMP-RM-splice variants alone and/or in combination from infection-induced or non-induced respiratory secretion samples were developed.

The preparation and development of the test kits of the present invention for measuring MMPs has been described in patent US 5,736,341 which is herewith incorporated by reference. Even if said patent is restricted to the development of test kits for diagnosing periodontal diseases by aid of binding substance recognizing the active site of MMP-8, the man skilled in the art can use the information for developing corresponding test kits for measuring MMP-RMs of the present invention. Other methods and test kits useful as chair- or bed-side kits and applicable as on-field test kits are described in the following non-exhaustive list of patent publications: US 5,591,645, US 5,712,170, US 5,602,040, US 5,622,871, US 5,656,503, EP 149168, US 4,552,839, US 4,361,537, US 4,373,932, WO 86/04683, EP 154749, EP 7654, WO86/03839, EP 191640, EP 212599, US 4,552,839, EP 158746, EP 225054.

Any immunochemical test methods can in principle be used for diagnosing the severity of respiratory tract inflammation as well as for longitudinal or latitudinal screening of the progress of disease and effect of medical treatment. However, visual agglutination, flow-through and immunochromatographic methods are best suited for rapid chair or bed-side tests. It is possible to prepare test sticks or test strips, which contain several different antibodies in separate zones and with which it is possible to detect several MMPs and/or MMP-RMs simultaneously.

In a specific embodiment of the present invention, the physician or veterinarian can collect a specimen of a respiratory secretion sample representing whole lung or part of the lung by placing a absorbing strip or corresponding solid carrier in an endoscopic device and/or catheter. The test strip is allowed to absorb liquid from the respiratory tract, preferably for a standardized time. Finally, a dipstick-type test device can be so designed that it includes an absorbing end that is placed in contact with the sample source, which is absorbed directly into test device.

The feasibility and applicability of the methods and test kits of the present invention are described in the following examples describing the experiments performed and their results.

#### **Example 1**

##### **Sample collection methods in man**

##### **a. BAL performance and BALF treatment**

The BAL was performed via fiberoptic bronchoscope under local anesthesia. Five 20 ml-aliquots of 0.9% NaCl, warmed up to 37° C, were installed into the segmental bronchus with immediate aspiration back after each aliquot. From untreated/uncentrifuged BALF, 50  $\mu$ l was used for Trypan blue staining evaluating surveillance of the BALF cells, and 3-ml was used for cyto-centrifuge preparations. The rest of BALF was centrifuged for 15 min. at 500 g, the supernatant was divided and stored in 500  $\mu$ l portions at -70°C. The cellular pellet was stored as a cytocentrifuge-preparation at -70°C.

##### **b. Induced sputum**

An ultrasonic nebulizer, the reservoir of which was filled with 100 ml of sterile 3% saline, was used. After rinsing the mouth, induction of sputum was performed via inhalation of solution by the patient for 5 min. periods for up to 30 min. The patients collect their sputum samples in 50-ml sterile

tubes, which were kept in the ice during sample collection. The induced material was divided as follows: (i) an equal volume of dithiotrietol 10 % (Sputolysin; Behring Diagnostics Inc., Somerville, NY) was added to the sample, and the solution was mixed by vortex at room temperature for 15 min. After centrifugation at 500g for 20 min., the supernatant was separated and stored at 70°C, the cell pellet was suspended in PBS and cytocentrifuged. Alternatively, the following sample collection protocol can be used. A part of sputum sample was centrifuged at 50,000g for 60 min. at 4°C, and separated phases of supernatant were stored in aliquots at -70°C, whereas cellular pellet was subjected to the same treatment as described above.

#### c. Sputum

Sputum sample as a natural, unforced material from the airways, was coughed out by the patients after rinsing of the mouth. Collected sputum sample was treated as induced sputum samples, as described above.

#### Example 2

##### Sample collection methods in horses

Tracheal epithelial lining fluid (TELF) was collected by flushing the tracheobronchial wall of the respiratory tract with sterile physiologic saline (10 ml) using a catheter inserted through the biopsy channel of an endoscope (Raulo, S.M., et al., AJVR, 59(7): 818-823, 1998). The pool formed by flushing in the lower trachea was sucked back completely. The urea concentrations in blood serum and in the tracheal flush were analyzed in parallel and the dilution effect of saline corrected (Rennard, S.I., et al., J. Appl. Physiol., 60 (2): 532-538, 1986). By this method activities and concentrations in the undiluted tracheal epithelial lining fluid (TELF) could be determined. Also plane TELF can alternatively be sucked with catheter or other sampling devices.

Tracheal flush fluid (TFF) is obtained by flushing like TELF. The native tracheal flush fluid without urea correction was used for analysis and it represents both the amount of TELF in the respiratory tract as well as the enzymes in TELF. If the sampling method described above is used, 60-90 cm<sup>2</sup> of the lower tracheal floor is sampled. The predisposition of a certain area of respiratory epithelial surface on enzymatic tissue destruction depends both on quality (the action of degradative enzymes in TELF) and quantity of TELF.

Bronchoalveolar lavage fluid (BALF) was collected with 2.5 m long endoscope under sedation and local anesthesia of the respiratory tract. The endoscope was wedged to a bronchi and a lung lobe originating from a 0.8 cm diameter bronchi was flushed with 300 ml of sterile saline. Cells were separated by centrifugation and the supernatant used for analysis. The cells were suspended and cytocentrifuged for clinical diagnostics.

### Example 3

Expression of MMP-9 in induced sputum (IS) in healthy controls and diseased individuals.

Induced sputum (IS) samples were collected and standardized according to the methods described above in Example 1. MMP-9 was identified and quantitated by Western blotting (Sorsa, T., et al., Ann. N.Y. Acad. Sci., 732: 112-131, 1994; Westerlund, U., et al., J. Dent. Res., 75 (8): 1553-1563, 1996) from healthy controls (n=14) and of patients with bronchial asthma (n=19). The prevalence of molecular forms was quantified by use of an image analysis and processing system (Bio-Rad Model GS-700) and expressed as arbitrary units.

Figure 1 shows a diagram of densitometric scanning analysis of Western blot of MMP-9 of induced sputum in patients with bronchial asthma compared to healthy controls. Increased amounts of total MMP-9 immunoreactivities are seen in sputum of severe



and mild asthma patients relative to controls. It is noteworthy, that in asthmatic sputum pathologically elevated MMP-9 is consistently activated.

#### Example 4

**Expression of MMP-9 in BAL fluid in healthy controls and diseased individuals.**

Bronchoalveolar lavage fluid (BALF) of healthy controls (n=14) and patients with bronchial asthma (n=45), COPD (n=23) and bronchitis (n=48) were obtained according to above described method. MMP-9 was identified and quantitated with Western blotting according to Example 3. The molecular forms were quantified by use of an image analysis and processing system (Bio-Rad) and expressed as arbitrary units.

Figures 2 and 3 show diagrams of densitometric scanning of Western blots of MMP-9 of BAL fluid in patients with bronchial asthma, bronchitis and chronic obstructive pulmonary disease compared to healthy controls. Increased amounts of total MMP-9 immunoreactivities are seen in BALF of asthma (Figure 2) bronchitis and COPD (Figure 3). Pathologically elevated MMP-9 is consistently activated. Furthermore successful treatment of asthma with corticosteroids reduces the pathologically elevated MMP-9 amounts and its degree of activation is close to values seen in healthy controls (Figure 2).

#### Example 5

**Expression of MMP-8 in induced sputum (IS) in healthy controls and diseased individuals**

Induced sputum (IS) samples were collected according to Example 1. MMP-8/collagenase-2 was identified and quantitated by Western blotting (Sorsa, T., et al., Ann. N.Y. Acad. Sc., 732: 112-131, 1994; Golub, L. M., et al., Infl. Res., 46 (8): 310-329, 1997); Lauhio, A., et al., Clin. Exp. Immunol., 98 (1): 21-28, 1994) from healthy controls (n=14) and from

patients with bronchial asthma (n=19) and bronchiectasis (n=8). The molecular forms were quantified by use of an image analysis and processing system (Bio-Rad) and expressed as arbitrary units.

Figures 4 and 5 show diagrams of densitometric scanning of Western blots of MMP-8 of induced sputum in patients with bronchial asthma and bronchiectasis compared to healthy controls. Increased amounts of MMP-8/collagenase-2 are seen in sputum of patients with asthma (Figure 4) and bronchiectasis (Figure 5) compared to controls. It is noteworthy that pathologically elevated MMP-8 is consistently activated.

#### Example 6

Expression of MMP-8 in BAL fluid in healthy controls and diseased individuals

Bronchoalveolar lavage fluid (BALF) of healthy controls (n=14) and patients with bronchial asthma (n=45) and bronchiectasis (n=48) were collected according to Example 1 and the quantitative Western blot was carried out according to Example 5.

Figures 6 and 7 show diagrams of densitometric scanning of Western blots of MMP-8 of BAL fluid in patients with bronchial asthma and bronchiectasis before and after treatment as compared to healthy controls. Increased amounts of MMP-8 are seen in BALF of asthma (Figure 6) and bronchiectasis (Figure 7) relative to controls. Noteworthy pathologically elevated MMP-8 is consistently activated, successful treatment of asthma with corticosteroids reduces pathologically elevated MMP-8 and its degree of activation close to the values seen in healthy controls.

#### Example 7

Measurement of human MMP-8 by immunofluorometric assay

The amounts of MMP-8 in induced sputum samples, collected as

described in Example 1, from patients with bronchial asthma (n=16), bronchiectasis (n=4) and healthy controls (n=9) were analysed by a time-resolved immunofluorometric assay (IFMA) utilizing two monoclonal antibodies 8706 and 8708 to human MMP-8 (Medix Biochemica, Kauniainen, Finland; Hanemaaijer, R., et al., J. Biol. Chem., 272 (50): 31504-31509, 1997). 8708 was coated onto microtitration wells, and 8706 labeled with an europium chelate (Hemmilä, I., et al., Anal. Biochem., 137: 335-343, 1984) was used as the tracer. Sample and assay buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 5 mM CaCl<sub>2</sub>, 50 mM ZnCl<sub>2</sub>, and 0.5 % bovine serum albumin) were incubated in the coated wells for 1 hour, after which the wells were washed and filled with 100 µl of assay buffer containing the tracer antibody. After further incubation of 1 h the wells were washed and 100 µl of Enhancement solution (Wallac, Turku, Finland) was added to each well. The fluorescence was measured after 5 minutes with a 1234 Delfia Research Fluorometer (Wallac, Turku, Finland), and the samples were compared to standards of known concentration. The MMP-8 levels were expressed in ng/ml or µg/l.

Figure 8 shows elevated MMP-8 amounts measured by IFMA-assay utilizing monoclonal antibody specific for MMP-8 were seen in bronchiectasis and asthma patients relative to controls.

The increased amounts of MMP-8 detected in bronchiectasis and asthma sputum samples in relation to healthy controls by IFMA (in Example 7 and shown in Figure 8 were compared with the results obtained with a dip-stick test kit described in Example 8.

#### Example 8

Dip-stick results showing MMP-8 positivity in induced sputum samples in health and disease

Induced sputum (IS) samples were collected and standardized according to the methods described above. Presence of elevated

level of MMP-8/collagenase-2 was determined by a dip-stick test from healthy controls (n=9) and of patients with bronchial asthma, and bronchiectasis.

The dip-sticks were prepared as follows:

A narrow zone of a nitrocellulose strip (strip 1, approximately 60 x 250 mm) was coated with a monoclonal antibody against MMP-8 (clone 8706, Oy Medix Biochemica Ab). Coloured latex particles were coated with another MMP-8 antibody (clone 8708, Oy Medix Biochemica Ab). The coated latex particles were dried on a zone in the middle of a strip (strip 2, approximately 60 x 300 mm) of absorbing polyethylene material. The diameter of the latex particles was small enough that they could flow freely through the pores in both strip materials. The two strips were attached on a plastic backing (approximately 60 x 1000 mm) so that they were in a contact which allows capillary flow of sample liquid from strip 2 to strip 1 when the end of strip 2 is dipped into liquid. For absorption of excess liquid, a pad of filter paper was attached in contact with strip 1 opposite to strip 2. The dipstick constructed was used to perform the rapid MMP-8 test according to the following instructions.

Performance of the immunochromatographic MMP-8 test:

1. The samples were diluted 1:10 in buffer (50 mM HEPES, 200 mM NaCl, 1 mM  $\text{CaCl}_2$  x 2  $\text{H}_2\text{O}$ , 0.1 % albumin, pH 7,5)
2. One end of the dipstick (end of strip 2) was dipped into sample liquid and kept there until the liquid front has reached strip 1, and then removed from the sample.
3. During an incubation of 5 minutes the sample migrated in the strips and the latex particles were transferred with the liquid over the antibody-coated zone to the other end of the dipstick.

4. Strip 1 was inspected. If a coloured zone was formed, the result was interpreted as positive. The detection limit was approximately 40  $\mu\text{g/l}$  MMP-8 in a diluted sample.

Data obtained by the monoclonal antibody based MMP-8-dipstick-test was compared with MMP-8 IFMA analysis; increased levels of MMP-8 correlated well with MMP-8 dip stick analysis (Figure 9). A cut-off area around 500  $\mu\text{g/l}$  of MMP-8 was adjusted to differentiate active lung disease from controls. Positive dip-stick results were strongly/predominantly associated with bronchiectasis and bronchial asthma, which patients had pathologically elevated MMP-8 IFMA values (Figure 8).

#### Example 9

Concentration of MMP-8 in BALF fluid samples in healthy controls and diseased individuals

Bronchoalveolar lavage fluid (BALF) of healthy controls (n=9) and patients with bronchial asthma (n=26), COPD (n=17) and bronchiectasis (n=9) were collected according to Example 1. MMP-8/ collagenase-2 concentration was measured by IFMA (See Example 7).

Figure 10 shows elevated MMP-8 concentrations ( $\mu\text{g/l}$ ) in BAL fluid samples of patients with bronchial asthma, COPD and bronchiectasis in relation to healthy controls

#### Example 10

Expression of MMP-13 in induced sputum (IS) in healthy controls and diseased individuals

Induced sputum (IS) samples were collected as described in Example 1. MMP-13/collagenase-3 was identified and quantitated by Western blotting (Golub, L.M., et al., Infl. Res., 46 (8): 310-329, 1997) from healthy controls (n=14) and of patients with bronchial asthma (n=19). The molecular forms were

quantified by use of an image analysis and processing system (Bio-Rad) and expressed as arbitrary units.

Figures 11 and 12 show diagrams of densitometric scanning analysis of Western blots of MMP-13 of induced sputum in patients with bronchial asthma and bronchiectasis compared to healthy controls. Elevated MMP-13 amounts were seen in induced sputum from asthma patients. Noteworthy, pathologically elevated MMP-13 is consistently activated.

#### Example 11.

MT1-MMP measured by Western blotting from induced sputum

Induced sputum samples, collected as described in Example 1, were analysed using quantitative Western-blotting, as described in Example 3, for MT1-MMP. Elevated MT1-MMP immunoreactivities were shown in induced sputum samples from bronchial asthma patients (n=4) relative to healthy controls (n=6). Soluble form of elevated MT1-MMP was consistently activated in asthma patients samples.

Figure 13. Induced sputum samples were analysed using quantitative Western-blotting for MT1-MMP. Elevated MT1-MMP immunoreactivities were in induced sputum samples from bronchial asthma patients (n=4) relative to healthy controls (n=6); soluble form of elevated MT1-MMP was consistently activated in asthma patients samples.

#### Example 12.

NGAL measured by Western blotting from induced sputum

Induced sputum samples, collected as described in Example 1, were analysed using quantitative Western-blotting, as described in Example 3, for NGAL. Elevated NGAL immunoreactivities were found in induced sputum samples from bronchial asthma patients (n=4) relative to healthy controls (n=6).

Figure 14. Induced sputum samples were analysed using quantitative Western-blotting for NGAL. Elevated NGAL immunoreactivities were in induced sputum samples from bronchial asthma patients (n=4) relative to healthy controls (n=6).

### Example 13

#### Gelatinolytic activity in TELF in horses suffering from COPD

Expression of gelatinolytic activity by gelatin zymography in tracheal epithelial lining fluid (TELF) in health and disease epithelial lining fluid from tracheobronchial area (TELF) of the respiratory tract of healthy horses (n=15) and horses suffering from COPD (n=17) was collected by flushing with a catheter through the biopsy channel of an endoscope and prepared according to the methods used previously by the inventors (Raulo, S.M., et al., A.V.J.R. 59(7): 818-823, 1998).

The TELF was collected by flushing under visual control of an endoscope using 10 ml of physiologic sterile saline. The pool formed by flushing in the lower trachea was sucked back completely. The urea concentrations in blood serum and the tracheal flush were analysed in parallel and the dilution effect of saline corrected (Rennard, S.I., et al., J. Appl. Physiol., 60: 532-538, 1986). By this method gelatinolytic activity in the nondiluted epithelial lining fluid could be determined. Zymography was carried out by using gelatin as substrate (Sepper, R., et al., Chest, 106(4): 1129-1133, 1994). The TELF samples (dilution 1:6 as assessed by the urea method) were separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Sorsa, T., et al., J. Biol. Chem., 272: 21067-21074, 1997) using 8-10 % cross-linked gels containing 1mg/ml porcine skin gelatin as substrate. After electrophoresis the gels were washed to remove SDS and thereafter incubated in Ca++ and Zn++ enriched buffer for 16 hours at 37°C. During incubation gelatin was degraded by the

gelatinolytic enzymes and the degradation at different molecular weight areas was visualized with Coomassie brilliant blue. The degradation was visualized as bright bands against blue background. The level of degradation was densitometrically quantified using an image analysis and processing system Cream<sup>TM</sup>, Kem-En-Tek, Copenhagen, Denmark. The gelatinolytic activity of each sample was compared to the activity of the standards of the same electrophoretic run. Gelatinolytic activities were expressed in relation to the standard (equine neutrophil MMP-9) within the same electrophoretic run and expressed as densitometric units. All gelatinase activities increase significantly in COPD in relation to healthy and diseased horses. Active forms of gelatinase products are detected only in increased amounts from the respiratory secretions of COPD horses.

Figure 15 shows a diagram presenting gelatinolytic activities of TELF (average  $\pm$ sd) in healthy horses and in horses suffering from COPD. All activities increase significantly. Active products are detected only in the respiratory secretions of COPD horses. Compl = complexed MMP-RM having gelatinolytic activity; Pro = proMMP-9; Act = activated gelatinolytic MMP-9 and other activated gelatinolytic MMPs

#### Example 14

Expression of MMP-9 in tracheal epithelial lining fluid (TELF) in health and disease.

Tracheal epithelial lining fluid was collected and standardized according to the methods described in Example 2. MMP-9 was identified and quantitated by Western blotting (Sorsa, T., et al., J. Biol. Chem., 272 (34): 21067-21074, 1997) from TELF of healthy horses (12) and of horses suffering from COPD (n=13). The intensity of molecular forms was densitometrically quantified using an image analysis and processing system Cream<sup>TM</sup>, Kem-En-Tek, Copenhagen, Denmark. Activated MMP-9 fragments reflecting MMP-9 activation are only



detected in COPD. Total immunoreactivity as well as immunoreactivities of all fractions of MMP-9 are increased significantly ( $p < 0.05$ - $P < 0.001$ ) in COPD TELF. Fractions reflecting activation increased most significantly.

Figure 16 shows a diagram of a densitometric scanning analysis of the amounts of MMP-9 in respiratory epithelial lining fluid (TELF) of healthy horses and horses suffering from COPD as evaluated by scanning the Western blots. Compl = complexes of MMP-9; Pro = proMMP-9; Act = Active MMP-9; Fragm = Fragments of MMP-9 reflecting activation

#### Example 15

Expression of MMP-9 in tracheal flush fluid (TFF) in healthy controls and diseased individuals

Tracheal flush was collected as in Example 2. The native tracheal flush fluid (TFF) represents the total pool of RS of 60-90 cm<sup>2</sup> of lower tracheal floor. This TFF originates from the bronchial and alveolar region of the lung. In lung diseases the amount of epithelial lining fluid increases and thus the enzymatic activity of the original flush represents both the activity in TELF as well as the amount of TELF in the respiratory tract. The predisposition of a certain area of epithelial surface on enzymatic degradation depends both on quality (the activity of degradative enzymes in TELF) as well as on the quantity of TELF.

The MMP-9 content by Western blotting was assessed using the same protocol as in Example 14. The results of healthy horses and horses suffering from COPD are shown in Figure 17. Activated MMP-9 amounts are detected in COPD. Total immunoreactivity as well as immunoreactivities of all fractions of MMP-9 are increased significantly ( $p < 0.05$ - $P < 0.001$ ) in COPD TFF.

Figure 17 shows a diagram of a densitometric scanning analysis

of the amounts of MMP-9 in native tracheal flush fluid of healthy horses and horses suffering from COPD as evaluated by scanning the Western blots. Compl = complexes of MMP-9; Pro = proMMP-9; Act = Active MMP-9; Fragm = Fragments of MMP-9 reflecting activation

#### Example 16

MMP-9 immunoreactivity in bronchoalveolar lavage (BALF) fluid in health and disease.

Bronchoalveolar lavage fluid was collected with a 2.5 m long endoscope under sedation and local anesthesia of the respiratory tract from healthy horses and from horses suffering from COPD. The endoscope was wedged to a bronchi and a lung lobe originating from a 0.8 cm diameter bronchi was flushed with 300 ml of sterile saline. Cells were separated by centrifugation and the MMP-9 immunoreactivity of the supernatant was determined by Western blotting as described in Example 14. The total amounts of activated MMP-9 fractions ( $p < 0.001$ ) and the separate active MMP-9 immunoreactive fractions ( $p < 0.01$ ) are elevated significantly in COPD when compared to healthy controls.

Figure 18 shows a diagram representing amounts of MMP-9 in a cell-free BAL fluid of healthy horses and horses suffering from COPD. The total amount of activated MMP-9 and MMP-9 fragments resulting from MMP-9 activation are elevated in COPD when compared to healthy.

#### Example 17

Expression of MMP-8 in TELF in healthy controls and diseased individuals

Tracheal epithelial lining fluid was collected and standardized according to the methods described in Example 2. MMP-8 was identified and quantitated by Western blotting (Golub, L.M., et al. Infl. Res., 46 (8): 310-329, 1997) from TELF of

healthy horses (n=8) and of horses suffering from COPD (n=11). The intensity of molecular forms was quantified using an image analysis and processing system Cream<sup>TM</sup>, Kem-En-Tek, Copenhagen, Denmark. A clear increase in both fibroblast-type ( $p < 0.001$ ) and polymorphonuclear leucocyte-type ( $p < 0.05$ ) MMP-8 was detectable in equine COPD TELF. Most prominent increase ( $p < 0.001$ ) was detected in MMP fragments or lower molecular weight species, end result reflecting activation of both fibroblast and PMN-type MMP-8.

Figure 19 shows a diagram presenting amount of MMP-8 (average  $\pm$  SD) in respiratory epithelial lining fluid (TELF) of healthy horses and horses suffering from COPD as evaluated by scanning the Western blots. In the Figure proPMN-8 indicates polymorphonuclear leucocyte-type MMP-8; proFB-8 fibroblast-type MMP-8, actFB-8 active fibroblast-type MMP-8, and Fragments mean degradation products due to activation of MMP-8. A clear increase of pro- and active forms of MMP-8 in equine TELF was detectable due to COPD in relation to healthy controls. Most prominent was the increase in fragmentation products resulting from activation of MMP-8 in COPD TELF relative to healthy TELF.

#### Example 18

##### Expression of MMP-13 in TELF in health and disease

Tracheal epithelial lining fluid was collected and standardized according to the methods described in Example 2. MMP-13 was identified and quantitated by Western blotting (Golub, L.M., et al., Infl, Res. 46: 310-317, 1997). N.Y. Acad. Sci., 732: 112-131, 1994; from TELF of healthy horses (n=9) and of horses suffering from COPD (n=11). The intensity of molecular forms was quantified using an image analysis and processing system Cream<sup>TM</sup>, Kem-En-Tek, Copenhagen, Denmark. A clear increase in both proMMP-13 ( $p < 0.01$ ) and degradation fragments of MMP-13 ( $p < 0.05$ ) were detected from equine COPD TELF in comparison to healthy TELF.

Figure 20 shows a diagram presenting amount of MMP-13 (average  $\pm$  SD) in respiratory epithelial lining fluid (TELF) of healthy horses and horses suffering from COPD as evaluated by scanning the Western blots. In the Figure proMMP-13 is the proform of MMP-13 and Fragments mean degradation products related /reflecting activation of MMP-13. A clear increase in proMMP-13 and even a higher increase in activation/fragmentation products resulting from activation of MMP-13 were detected in COPD TELF compared with healthy TELF.